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(54) Title: EMBRYONIC STEM CELL-LIKE CELLS

(57) Abstract

A process for preparing embryonic stem cell-like cells ("ES-like cells") is described. The process includes providing a source of primordial germ ("PG") cells, and a source of feeder cells of a suitable type; contacting the PG cells with the feeder cells; culturing the PG cells in a culture medium; and identifying and isolating ES-like cells from the culture medium; wherein the culture step is performed in the absence of exogenous growth factors and/or exogenous cytokines.

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EMBRYONIC STEM CELL-LIKE CELLS

The present invention relates to a process for preparing embryonic stem cell-like cells and embryonic stem cell-like cells produced thereby.

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) cells of blastocysts and retain their pluripotency after *in vitro* culture. ES cells can thus be cultured and manipulated *in vitro* and then returned to the embryonic environment to contribute normally to all tissues including the germ line. Not only can ES cells propagated *in vitro* contribute efficiently to the formation of chimaeras, including germ line chimaeras, but in addition these cells can be manipulated *in vitro* without losing their capacity to generate germ line chimaeras.

However, whilst the technologies of the derivation and experimental utilisation of ES cells from the mouse are well established, the extension of these technologies from the mouse to major domestic species has proved to be extremely difficult. Whilst Applicant does not wish to be restricted by theory this may relate to two specific differences in the development of murine and ungulate embryos. Firstly, the isolation of mouse ES cells may have been facilitated by the timing of differentiation of the blastocysts before the onset of delamination of primary endoderm. Secondly, development of the inner cell mass differs significantly between murine and other mammalian species; in mouse embryos proliferation of the inner cell mass occurs rapidly, whereas in many other mammalian embryos the inner cell mass forms a mitotically quiescent embryonic disc.

Subsequently, researchers have attempted to isolate ES cells from domestic animals using variations of the classical methods. For example, in International Application PCT/GB89/01103, production of pluripotential embryonic stem cells derived from porcine and bovine species was claimed. However, since publication of this application, evidence of the cells' ability to contribute to chimeras has not been forthcoming.

Primordial germ (PG) cells are the progenitors of the gametes in developing vertebrates. It is thought that PG cells arise quite early in development, perhaps in the preimplantation embryo. As the bearers of genetic information between generations they are shielded from differentiative influences by being segregated in the yolk sac of the developing foetus until such time as

they receive an (as yet unknown) signal to migrate to the gonadal ridges, the precursors of the gonads. Upon arrival at the gonadal ridges, at approximately 30 days of gestation in cattle, they enter a phase of mitosis followed by meiotic division. The timing and duration of these phases varies between species and they may be interspersed with periods of quiescence also of varying length. PG cells are theoretically totipotent, that is, they have the potential to differentiate into all tissue types under the right conditions (usually fertilisation following meiosis but parthenogenesis is also possible). The presence of the homeobox transcription factor Oct-4 in PG cells but in no other cells of the developing foetus highlights this potential.

In recent years, techniques have evolved for the isolation and long term culture of PG cells from mouse foetuses. Under favourable conditions the PG cells of certain mouse strains may be induced to revert or convert to embryonic stem cell-like cells, immortal totipotent cell lines of great utility for the production of transgenic mice and the study of differentiative and developmental processes. However, these cells could only be maintained in culture if fibroblast growth factor ("FGF") was added to the culture medium and in the presence of steel factor and leukaemia inhibitory factor (LIF).

Whilst PG cells from species other than the mouse have been isolated and maintained in short-term culture, the absence of specific biochemical markers for the PG cells as exist for the mouse has made difficult the unambiguous identification of PG cell candidate cells.

It would be a significant advance in the art if embryonic stem cells or embryonic stem cell-like cells could be produced from vertebrate species generally including livestock such as cattle, sheep, pigs and poultry, and primates such as humans.

It would also be a significant advance in the art if the isolation of PG cells and the culture and identification of PG-derived embryonic stem cell-like cells could be simplified.

It is accordingly an object of the present invention to overcome or at least alleviate one or more of the difficulties and deficiencies of the prior art.

Accordingly, in a first aspect of the present invention there is provided a process for preparing embryonic stem cell-like cells (ES-like cells) which process

includes

providing

a source of PG cells; and

a source of feeder cells of a suitable type;

contacting the PG cells with the feeder cells;

culturing the PG cells in a culture medium; and

identifying and isolating ES-like cells from the culture medium;

wherein the culture step is performed in the absence of exogenous growth factors and/or exogenous cytokines.

It has been surprisingly found that culturing PG cells in the presence of feeder cells of a suitable type enables ES-like cells to be cultured in a culture medium which lacks exogenous cytokines such as LIF and steel factor and/or exogenous growth factors such as FGF. By "exogenous growth factors" is meant growth factors which are added to the culture medium. By "exogenous cytokines" is meant cytokines which are added to the culture medium. The term "added" includes genetic manipulation of the feeder cells to produce cytokines and/or growth factors. Whilst applicant does not wish to be restricted by theory, it is thought that feeder cells of a suitable type may be providing factors that are required for culture of the PG cell-derived ES-like cells.

The source of PG cells utilised in the process according to this aspect of the present invention may be of any suitable type. Preferably, the PG cells are from a vertebrate species. More preferably, the PG cells are from primates or domestic livestock, including ruminants, pigs and poultry. It is preferred that the foetus is of a gestational age such that the PG cells have entered the gonadal anlagen and are in the proliferative or mitotic growth phase. In the case of cattle this corresponds to between approximately 29 and 60 days gestation. The gonadal ridge or gonads from a foetus may be used as a source of PG cells. The gonadal ridges or gonads may be dissected, washed and disaggregated for use in the process of the present invention. In this case, it is preferred that the gonadal ridges or gonads are from a foetus of gestational age between approximately 29 and 35 days. Alternatively, the gonadal ridges or gonads may be retained at least partially intact for culture. In this case, it is preferred that the gonadal ridges or gonads are from an embryo of gestational age between

approximately 35 and 55 days. The gonadal ridges or gonads are preferably punctured a plurality of times for culture.

Accordingly, in a preferred form of this aspect of the invention there is provided a process for preparing ES-like cells which process includes

providing

at least partially intact gonadal ridges or gonads from an embryo of gestational age between approximately 35 and 55 days; and

a source of feeder cells of a suitable type;

contacting the gonadal ridges or gonads with the feeder cells;

culturing the gonadal ridges or gonads in a culture medium; and

identifying and isolating ES-like cells from the culture medium;

wherein the culture step is performed in the absence of exogenous growth factors and/or exogenous cytokines.

In the process according to the first aspect of the invention the feeder cells are of any type suitable to enable culture of ES-like cells in the absence of exogenous growth factors and/or exogenous cytokines. Preferably, the PG cells and feeder cells are from homologous species. Preferably, the feeder cells are derived from an embryo or foetus. More preferably the feeder cells are obtained from an embryo or foetus of gestational age approximately that of the experimental tissue from which the PG cells are derived. Preferably the feeder cells are fibroblasts, more preferably embryonic fibroblasts. Preferably, the feeder cells are from a vertebrate species. More preferably, the feeder cells are from humans or domestic livestock, including ruminants. A ruminant fibroblast cell line may be used. An ovine or bovine fibroblast may be used. The feeder cells may be mitotically inactivated prior to use. The feeder cells may form a confluent feeder layer for the PG cells or may provide a conditioned medium.

Accordingly, in a preferred form of this aspect of the invention there is provided a process for preparing ES-like cells which process includes

providing

a source of PG cells; and

a source of homologous embryonic fibroblasts;

contacting the PG cells with the homologous embryonic fibroblasts;

culturing the PG cells in a culture medium; and

identifying and isolating ES-like cells from the culture medium;

wherein the culture step is performed in the absence of exogenous growth factors and/or exogenous cytokines.

In the process according to the first aspect of the invention the contacting step may be undertaken in any suitable manner. Preferably, the contacting step is undertaken in a standard tissue culture dish such as a 4-well tissue culture dish.

The culture step may be conducted in any suitable medium. The addition to the medium of cytokines such as LIF and steel factor and/or growth factors such as FGF is not required for isolation of ES-like cells according to the present invention. Preferably, the culture step is conducted in a conventional embryonic stem cell medium. More preferably the culture medium may be selected from Eagle's medium or modifications or equivalents thereof such as Dulbecco's, DMEM or Glasgow's modified Eagle's medium. Such medium may be supplemented with foetal calf serum or other suitable serum. The culture step is preferably conducted at a slightly elevated temperature, e.g. in the range of from 35°C to 40°C. Preferably the culture step continues for approximately 1 to 10 days, more preferably approximately 5 to 7 days.

ES-like cells may be identified by one or more of the following features:

1. relatively large size;
2. prominent vesicles;
3. non or weakly adherent;
4. smooth surface;
5. alkaline phosphatase staining;
6. spiky processes;
7. propensity to aggregate.

ES-like cells may be removed from the culture medium transferred to fresh culture medium and feeder layer. The ES-like cells are capable of multiple passage in this manner. The ES-like cells may be successfully frozen and thawed. The ES-like cells may be purified from the feeder layer or from contaminating stromal cells by any suitable means. A FACS sorter employing labelled antibodies specific for cell surface markers has been found to be

suitable.

In a further aspect of the present invention, there is provided an ES-like cell prepared as described above. Preferably, the ES-like cell is a vertebrate ES-like cell. More preferably, the ES-like cell is a primate or domestic livestock ES-like cell.

Utilising the process according to the present invention, a number of bovine cell lines have been isolated. Accordingly, in a preferred form of this aspect of the present invention there is provided bovine cell lines PGC1, PGC12, PGC13, PGC15, PGC16, PGC17, PGC18 and PGC 19 as hereinafter described.

In order to further characterise the ES-like cells, an ES-like cell specific assay has been developed. Accordingly in a further aspect of the present invention there is provided a method for testing ES-like cells, which method includes

providing

a source of putative ES-like cells; and

a non-labile marker;

incubating the putative ES-like cells and the marker to form labelled putative ES-like cells;

injecting the labelled putative ES-like cells into a morula or early blastocyst;

culturing the morula or early blastocyst for a sufficient time to enable proliferation of the grafted cells; and

subjecting the embryo thus produced to an assay to detect the marker.

The non-labile marker may be of any suitable type. Preferably, the non-labile marker is a fluorescent marker such as fluorescein isothiocyanate ("FITC") or a marker such as bromodeoxyuridine ("BrDU"). Where a fluorescent marker is used, a fluorescent or confocal microscope may be used to monitor the strength and distribution of fluorescence. It will be understood that if the cells proliferate, the fluorescence will be distributed amongst the daughter cells in proportion to the degree of proliferation. Moreover, concentration of fluorescence in the inner cell mass (ICM) is a further indication of an ES-like cell.

The morula or early blastocyst may be of any suitable type. Preferably, the morula or early blastocyst is from a vertebrate. More preferably, the morula or

early blastocyst is from a domestic animal including ruminants or a primate.

In a still further aspect of the present invention, the genetic material of the ES-like cells prepared as described above may be modified. For example, homologous recombination may be used for "gene knockout", gene "knockup", "hit and run" and other gene modifications. The use of "Yeast Artificial Chromosomes", mammalian artificial chromosomes and other gene constructs for transfection is contemplated.

Accordingly, in a further aspect of the present invention there is provided an ES-like cell prepared by a process as described above and including modified genetic material. Preferably, the ES-like cell is a vertebrate ES-like cell. More preferably, the ES-like cell is a primate ES-like cell or a domestic livestock ES-like cell.

In a still further aspect of the present invention there is provided a method for preparing chimaeric animals utilising ES-like cells prepared by a process as described above, and chimaeric animals produced by said method, including transgenic progeny of said animals generated by known techniques. The production of chimaeric animals may be achieved, for example, by incorporating ES-like cells into embryos by blastocyst injection, morula injection or morula aggregation, and transferring the treated embryos to primed recipient females. The contribution of grafted cells to the early gestation conceptus may be established by, for example, DNA fingerprinting techniques, polymerase chain reaction (PCR) or transfected markers. *In vitro* testing for evidence of the incorporation of ES-like cells into the embryo may require labelling of the type discussed above.

Accordingly, in this aspect of the present invention there is provided a method for preparing a nuclear transplantation embryo, which method includes

providing

an enucleated oocyte, and

an ES-like cell prepared by the process as described above; and

introducing the ES-like cell into the enucleated oocyte to reconstitute the genome and produce a nuclear transplantation embryo.

In this aspect of the invention there is also provided a method for preparing chimaeric animals, which process includes

providing

a nuclear transplantation embryo as described above, and

a recipient female; and

transferring the nuclear transplantation embryo to the recipient female.

Preferably, the ES-like cell is a vertebrate ES-like cell. More preferably, the ES-like cell is a primate or domestic livestock ES-like cell.

The enucleated oocyte may be enucleated by any suitable technique. Preferably, the enucleated oocyte is enucleated utilising micromanipulation techniques.

The enucleated oocyte may be reconstituted, for example via micromanipulation, aggregation or other techniques. Preferably, electrofusion techniques are utilised to reconstitute the genome.

The nuclear transplantation embryo may be transferred into a recipient female, preferably a primed recipient female, utilising known techniques.

ES-like cells according to the present invention provide a route for the generation of transgenic animals. A gene of interest can be introduced and its integration and expression characterised *in vitro*. The effect of the introduced gene on the ES-like cell growth can then be studied *in vitro* and the characterised ES-like cells, having a novel introduced gene, can be efficiently introduced into embryos by blastocyst injection or embryo aggregation and the consequences of the introduced gene on the development of the resulting transgenic chimaeras monitored during pre- and post-natal life. The site in the ES-like cell genome which the introduced gene integrates can be manipulated leaving the way open for gene therapy, gene knock-up, gene targeting and gene replacement.

ES-like cells according to the present invention can be used to identify factors influencing differentiation. Additionally, ES-like cells may serve as a vector for the transfer of desirable genes into the vertebrate genome. For example, by using ES-like cells from elite farm animals as the source of the genetic sequence, herd quality may be cheaply and rapidly increased. Furthermore, ES-like cells according to the present invention may be used as a source of differentiated cell lineages, eg. neuronal or haematopoeitic stem cells. Induced differentiation into a desired cell lineage enables the construction of artificial organs for transplantation or production *in vitro* of therapeutic drugs.

Transgenic animals according to the present invention, may also serve as the source of commercial quantities of clinically valuable substances.

The present invention will now be more fully described with reference to the accompanying drawings and examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention disclosed above. In particular, the description following should not be taken as a restriction on the invention either with regard to species or with regard to application.

In the figures:

Figure 1 shows colonies of PG cell derived bovine ES-like cells on foetal bovine fibroblasts (x 50 phase contrast).

Figure 2 shows colonies of PG cell derived ES-like cells exhibiting ES-like morphology growing on a layer of foetal bovine fibroblasts (x 125 phase contrast).

Figure 3 shows a colony of PG cell derived ES-like cells growing on foetal bovine fibroblasts. The aged colony (centre of photograph) has turned brown in the manner of murine aged ES cells (x 125 phase contrast).

Figure 4 shows a colony of PG cell derived ES-like cells at passage No. 6. The colony exhibits positive staining for presence of alkaline phosphatase (x 125 phase contrast).

Figure 5 shows a cystic embryoid body spontaneously formed from aggregated PG cell derived ES-like cells at passage No. 5 (x 400 phase contrast).

Figure 6A shows cells in the vicinity of gonadal tissue after 24 hour culture: Primary isolation of bovine PG cell derived ES-like cells from foetus of approx. 45-50 days gestation. A clump of PG cell derived ES-like cells (large cells in centre of photograph) can be seen adjacent to the gonad (x 200).

Figure 6B shows cells in the vicinity of gonadal tissue stained for alkaline phosphatase after 3 days culture. PG cell derived ES-like cells are stained for alkaline phosphatase (AP). Stromal outgrowth from gonad expresses no AP (x 100).

Figure 6C shows cells from the vicinity of gonadal tissue stained for alkaline phosphatase following subculture. Clumps of PGCs and individual cells were transferred to a new feeder layer of bovine embryonic fibroblasts (BEF).

AP-positive cells proliferate in culture (x 100).

Figure 6D shows a feeder layer of BEF cells stained for alkaline phosphatase. This is an AP-negative control (x 100).

Figure 7A shows ES-like cells after 7 days culture: Isolation of ES-like cells from bovine foetus of approximately 45-50 days gestation. After 7 days in culture PGCs form clumps (x 100).

Figure 7B shows ES-like cells after 14 days culture. Over days 7-14 cells proliferate extensively and form large clumps (x 100).

Figure 7C shows spiky processes on the surface of ES-like cells (x 200).

Figure 7D shows spiky processes on the surface of ES-like cells that have been transferred to a gelatinised tissue culture dish without a feeder layer (x 200).

EXAMPLE 1

Production of bovine embryonic stem cell-like cell lines from primordial germ cells

The bovine ES-like cell lines PGC1, PGC12, PGC13, PGC15, PGC16, PGC17, PGC18 and PGC19 were isolated according to the following procedure.

The gonadal ridges from a bovine foetus of gestational age between 29 and 35 days (1.5 to 2 cm crown-rump length) are removed by sterile dissection using a dissecting microscope. The gonadal ridges are briefly washed in PBS and then minced using fine scissors in a small volume (about 200 µl) of trypsin/versene (0.25%/1mM). The sample is placed in a water bath at 37°C and allowed to incubate for 5 min. The digest is then triturated using a small volume micropipette until most of the tissue is fragmented into small clumps and single cells. Larger clumps are allowed to fall to the bottom of the tube and the supernatant is aspirated and transferred to one well of a 4-well tissue culture dish bearing a confluent feeder layer of mitotically inactivated bovine embryonic fibroblasts grown in conventional embryonic stem cell medium. The dish is placed in a humidified incubator and maintained at 39°C for up to one week without change of medium. Within this period, large round alkaline phosphatase positive colonies composed of small cells and similar in morphology to murine ES cell colonies appear (see Figures 1 and 2). Individual colonies may be selected for subculturing or more commonly, the entire culture is transferred to a 35 mm

feeder dish following aspiration of medium, rinsing with PBS and digestion with trypsin/versene. If not subcultured within 5 days the colonies become brown in colour and appear to differentiate (see Figure 3). Cells can be subcultured in this way for at least 5 passages while retaining ES-like morphology and ability to express alkaline phosphatase activity (see Figure 4). These qualities are not affected by freezing and subsequent thawing and reestablishment of the cultures. When removed from the feeder layer the cells differentiate into a number of cell types including fibroblastic, nerve and epithelioid varieties depending on culture surface and density of seeding (see Figure 3). If cultured in hanging drops, the cells will form embryoid bodies (see Figure 5).

Further evidence of the pluripotent potential of the cell lines PGC1, PGC12 and PGC13 is provided by the presence of certain homeobox transcription factors which have been shown to identify undifferentiated cells in the mouse. Messenger RNA from *Oct4*, *Oct6* and *Hes1* have been detected in these cells using the PCR technique (see Table 1). The *Oct6* marker is a marker previously only detected in mouse ES and embryonal carcinoma (EC) cells and is therefore indicative of the ES-like nature of these ruminant PG cell derived ES-like cell lines.

TABLE 1

Expression of *Oct*4, *Oct*6 and *Hes*1 Genes

in ES-like Cells and PGC

	*Oct*4	*Oct*6	*Hes*1
PG cell (+) (from references)	+	-	?
ES cells (from references)	+	+	+
From PCR			
M78*	+	-	+
M107*	+	+	+
M109*	+	+	+
PGC1	-	+	-
PGC12	+	+	+
PGC13	+	+	+
MBL-5 ES cells	+	+	+
SNL Bayler Bradley cells	-	-	-
DEPC H_2O	-	-	-

* Murine ES cells derived by conventional means

EXAMPLE 2

MATERIALS AND METHODS

Isolation and primary culture of PG cells

Bovine foetuses of between 2.5 and 3.3 cm in length (crown rump) are collected from slaughterhouse tissue or by surgical means, placed in phosphate buffered saline at 37°C and transported to the laboratory in a portable incubator. The gonadal analgen or gonads are removed and placed in 0.02% EDTA in PBS for 20 min at 39°C. The tissue is then placed in 4-well dishes (Nunc) precoated with 0.1% gelatin and bearing a feeder layer of mitotically inactivated bovine embryonic fibroblasts (mitomycin-c, Sigma) obtained from a foetus of gestational age approximating that of the experimental tissue. Treated gonads are then pricked numerous times with a 37g needle and dishes returned to a humidified incubator (39°C, 5% CO2, 95% air). The culture medium is DMEM + 10% FCS, 1mM mercaptoethanol, penicillin/streptomycin.

Cell identification and characterisation

Alkaline phosphatase in cultured cells was detected using a dedicated kit (Sigma). Medium was aspirated from culture dishes and cells washed once with phosphate buffered saline (PBS) without calcium or magnesium. The staining procedure was carried out without allowing the culture to dry at any time. Cell growth and morphology may be monitored using an inverted phase-contrast light microscope.

RESULTS

24 hrs after treatment, numbers of cells of differing types including nucleated erythrocytes, small stromal cells and cells identified as PG cells by their large size and prominent vesicles may be observed in the immediate vicinity of the gonadal tissue (Fig. 6A). These latter cells are non- or weakly adherent and possess a smooth surface. The cells adjacent to the gonads increased in number over the next 2 days and expressed alkaline phosphatase (AP) strongly (Fig. 6B). Stromal outgrowths from disrupted gonads are negative or only weakly AP positive (Fig. 6B). Cells surrounding the gonads may be transferred to fresh feeder layers and observed after 48 h in culture. AP staining of the passaged cells reveals that substantial proliferation is occurring and strong expression of AP maintained in approximately 80% of the weakly-adherent cells (Fig. 6C). The feeder layer exhibits no staining (Fig. 6D).

Long-term culture

When retained in culture without disturbance for 7 days or longer, the non-adherent cells continue to proliferate and colonies or aggregates of these cells appear, often at some distance from the gonad (Fig. 7A). Within 14 days the numbers of these cells increase from hundreds to some hundred thousand (Fig. 7B). Under higher magnification, numerous processes extending from the cell membrane may be observed in all cells. These processes are from 10μm to 40μm in length and are spiky rather than bleb-like (Fig. 7C). The cells have been passaged up to 4 times to achieve a population of around 2×10^6 while retaining their processes and propensity to aggregate. Cell numbers remain static after this time. When placed on a gelatinised dish without a feeder layer, proliferation is dramatically reduced and the processes become longer and more apparent (Fig. 7D). The addition of bFGF, SCF, LIF or combinations of these factors has

no observable effect upon cell proliferation, longevity in culture or propensity to differentiate. The cells may be frozen at -70C or in liquid nitrogen in a mixture of 10% dimethyl sulphoxide and 90% foetal calf serum. Upon thawing, more than 90% of the cells remain viable as determined by vital staining and metabolic activity following return to culture.

DISCUSSION

Cultured but not freshly isolated PG cells exhibit processes from their cell membranes. Whilst applicant does not want to be restricted by theory, it is postulated that the liberation of PG cells from the confines of the gonad is a signal for the PG cells to revert to their earlier status as migratory cells. This changed status and the presence of a feeder layer of embryonic fibroblasts provides a stimulus to proliferate and to exhibit the clumping behaviour typical of migrating murine PG cells. Cultured cells may be frozen and thawed retaining over 90% viability. They therefore may provide an alternative source of transferable genes for nuclear transfer purposes while simultaneously overcoming some of the limitations of the current technology vis: severely restricted numbers of usable blastomeres or inner cell mass cells and the inability to store unused cells. The availability of great numbers of identical totipotent cells allows the possibility of transgenesis on a scale presently impractical. Furthermore, the technology provides large numbers of female primordial germ cells, offering the possibility of their *in vitro* differentiation and maturation into oocytes for nuclear transfer purposes. This may eventually eliminate the necessity of collecting oocytes from abattoir carcasses for maturation and fertilisation.

EXAMPLE 3

A. Isolation and primary culture of PG cells

1. Derive PG cells from gonadal ridge/gonad of bovine embryo of gestational age 35-55 days. Incubate GR in 0.02% EDTA for 15min followed by pricking with 37G needle.

2. PG cells are not like mouse PG cells - do not exhibit blebbing. Individual PG cells are large and grainy largely non-adherent cells.

3. Bovine PG cells can be identified by intense alkaline phosphatase staining. Other cells of the gonadal ridge may stain positive for alkaline phosphatase but much more weakly. Stromal cells are adherent.

4. The GR is placed on a layer of inactivated bovine embryonic fibroblasts (BEF) growing in conventional ES medium. After 2-3 days ES-like cells are seen as individual cells or clusters surrounding the GR.

5. Up to 1×10^3 ES-like cells may be collected using a finely drawn plasteur pipette and transferred to a fresh dish bearing inactivated BEF. ES-like cells will proliferate slowly over 7-10 days to ca. 5×10^4 in number. The cells remain lightly or non adherent and remain strongly alkaline phosphatase positive.

6. The primary ES-like cells may be successfully frozen and thawed.

7. ES-like cells may be purified from the feeder layer or from contaminating stromal cells by the use of a FACS sorter employing labelled antibodies specific for cell surface markers.

B. Use of primary ES-like cells

1. Totipotent ES-like cells may be employed in the manner of embryonic stem cells to create chimaeric animals by blastocyst injection. Unlike embryonic stem cells they may also be used as the donor nucleus in nuclear transplantation into enucleated oocytes.

2. Foreign DNA can be introduced into ES-like cells for the creation of transgenic animals.

3. The ES-like cells can be induced to further proliferate and survive multiple passages whilst retaining totipotency. This is achieved by culturing the ES-like cells in the presence of suitable growth factors. Proliferation in this manner will make the application of transgenic techniques more efficient.

EXAMPLE 4

1. The differentiative potential of the ES-like cells is tested by injecting them into bovine blastocysts. Numbers of the injected embryos have been transferred to recipient cows and foetuses have been obtained from the resulting pregnancies. The DNA of the foetuses is analysed for the presence of genes from the injected ES-like cells to determine the extent of any contribution to the chimaeric foetal tissues.

2. ES-like cells according to the invention have been successfully fused with enucleated bovine oocytes. We can achieve cleavage and embryonic development using ES-like cells according to the invention.

3. The technique of inducing proliferation of ES-like cells is applicable to other non-rodent species. Preferably, this involves the use of a feeder layer of embryonic fibroblasts obtained from a foetus of the corresponding developmental age and species.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

CLAIMS:

1. A process for preparing embryonic stem cell-like cells ("ES-like cells") which process includes

providing

a source of primordial germ ("PG") cells, and

a source of feeder cells of a suitable type;

contacting the PG cells with the feeder cells;

culturing the PG cells in a culture medium; and

identifying and isolating ES-like cells from the culture medium;

wherein the culture step is performed in the absence of exogenous growth factors and/or exogenous cytokines.

2. A process according to claim 1 wherein the feeder cells and the PG cells are from homologous species.

3. A process according to claim 2 wherein the cytokines include one or more of leukaemia inhibitory factor ("LIF") and steel factor, and wherein the growth factors include fibroblast growth factor ("FGF").

4. A process according to claim 2 wherein the feeder cells are derived from an embryo or foetus.

5. A process according to claim 4 wherein the feeder cells are fibroblasts.

6. A process according to claim 5 wherein the feeder cells are obtained from an embryo or foetus of gestational age approximately that of the experimental tissue from which the PG cells are derived.

7. A process according to claim 6 wherein the embryo or foetus is of gestational age such that the PG cells have entered the gonadal anlagen and are in a proliferative or mitotic growth phase.

8. A process according to claim 7 wherein the source of PG cells is at least partially intact gonadal ridges or gonads from an embryo of gestational age between approximately 35 and 55 days.

9. A process according to claim 7 wherein the source of PG cells is disaggregated gonadal ridges or gonads are from a foetus of gestational age between approximately 29 and 35 days.

10. A process according to claim 1 wherein the PG cells are from primates or domestic livestock, including ruminants, pigs and poultry.

11. A process according to claim 1 wherein the culture step is for approximately 1 to 10 days, preferably for approximately 5 to 7 days.

12. A process according to claim 1 wherein ES-like cells are identified by the presence of one or more of the following:

(a) relatively large size;
(b) prominent vesicles;
(c) non or weakly adherent;
(d) smooth surface;
(e) alkaline phosphatase staining;
(f) spiky processes;
(g) propensity to aggregate.

13. A process according to claim 1, which process includes the further step of removing ES-like cells from the culture medium and culturing said cells in fresh culture medium including fresh feeder cells.

14. A process according to claim 13 wherein the further step is performed at least twice.

15. An ES-like cell prepared by the process of claim 1.

16. An ES-like cell according to claim 15 which is a primate or domestic livestock ES-like cell.

17. An ES-like cell according to claim 15 including modified genetic material.

18. A bovine cell line selected from PGC1, PGC12, PGC13, PGC15, PGC16, PGC17, PGC18 and PGC19 as hereinbefore described.

19. A method for preparing a nuclear transplantation embryo, which method includes

providing

an enucleated oocyte; and

an ES-like cell prepared by the process of claim 1; and

introducing the ES-like cell into the enucleated oocyte to reconstitute the genome and produce a nuclear transplantation embryo.

20. A method for preparing chimaeric animals, which process includes

providing

a nuclear transplantation embryo according to claim 16, and

a recipient female; and

transferring the nuclear transplantation embryo to the recipient female.

21. A method according to claim 19 or 20 wherein the ES-like cell is a primate or domestic livestock ES-like cell.

22. A method for testing ES-like cells, which method incudes

providing

a source of putative ES-like cells; and

a non-labile marker;

incubating the putative ES-like cells and the marker to form labelled putative ES-like cells;

injecting the labelled putative ES-like cells into a morula or early blastocyst;

culturing the morula or early blastocyst for a sufficient time to enable proliferation of the grafted cells; and

subjecting the embryo thus produced to an assay to detect the marker.

23. A method according to claim 22 wherein the non-labile marker is fluorescein isothiocyanate ("FITC") or bromodeoxyuridine ("BrDU").

24. A process for preparing ES-like cells substantially as hereinbefore described with reference to any one of the examples.

Fig.1.

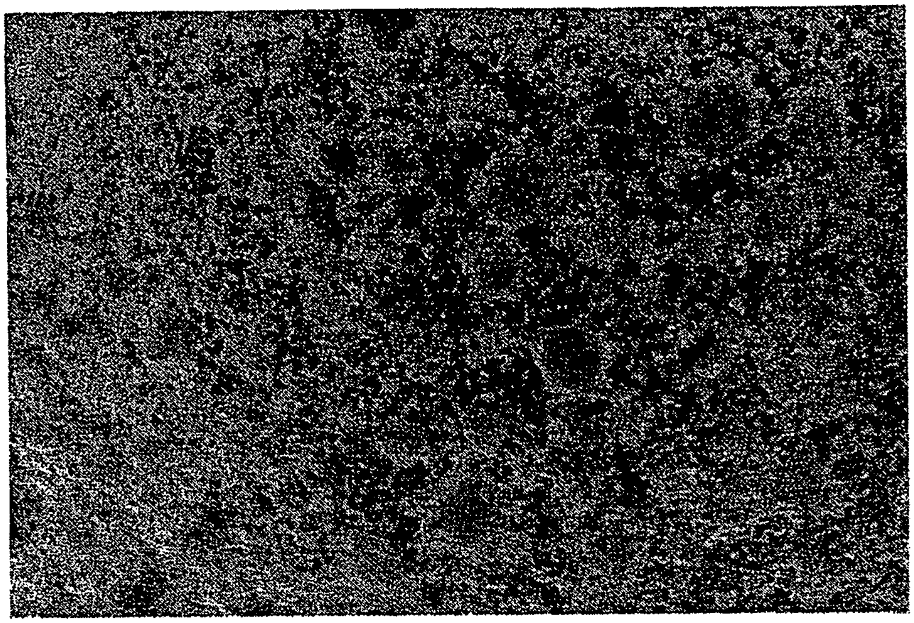


Fig.2.

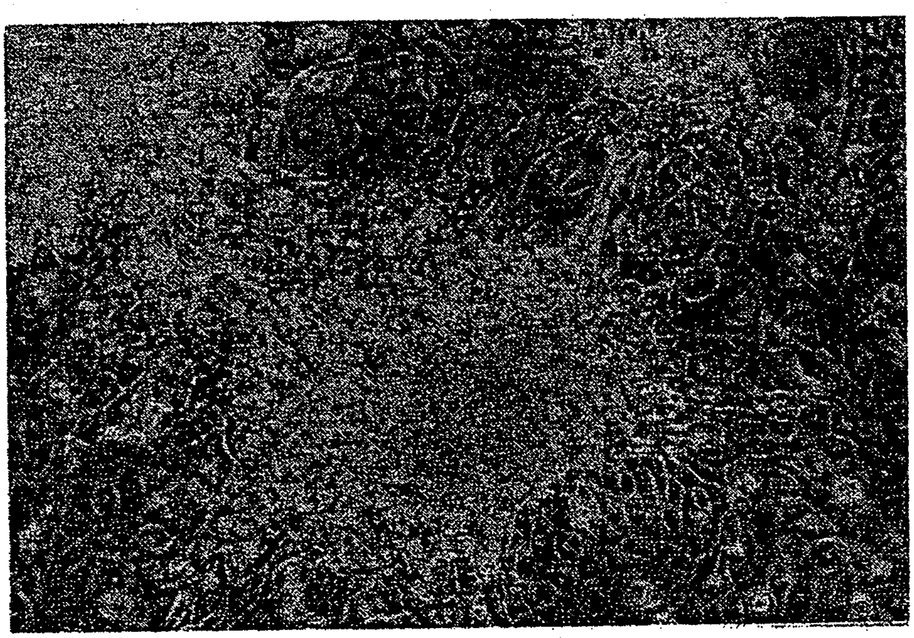


Fig.3.

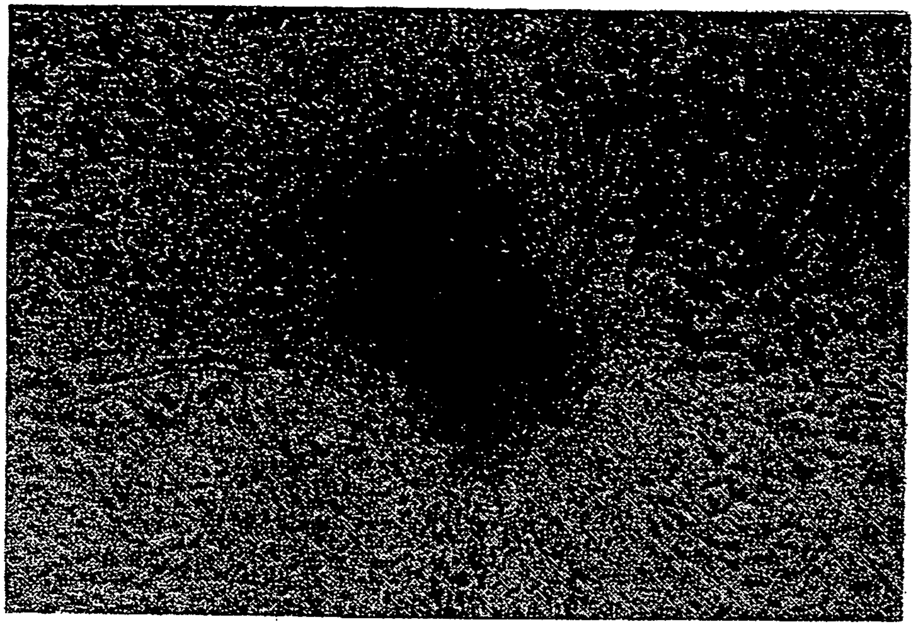


Fig.4.

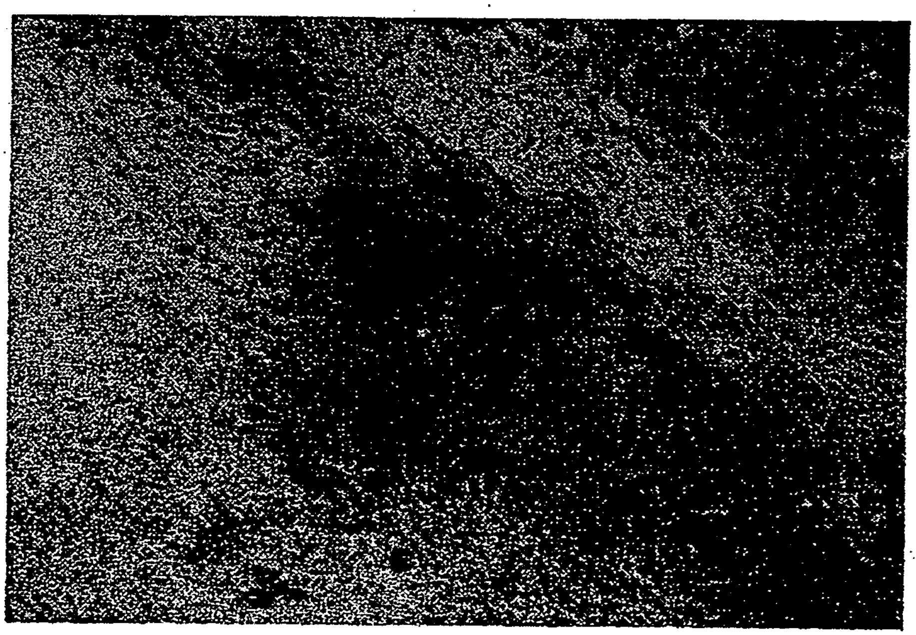


Fig.5.

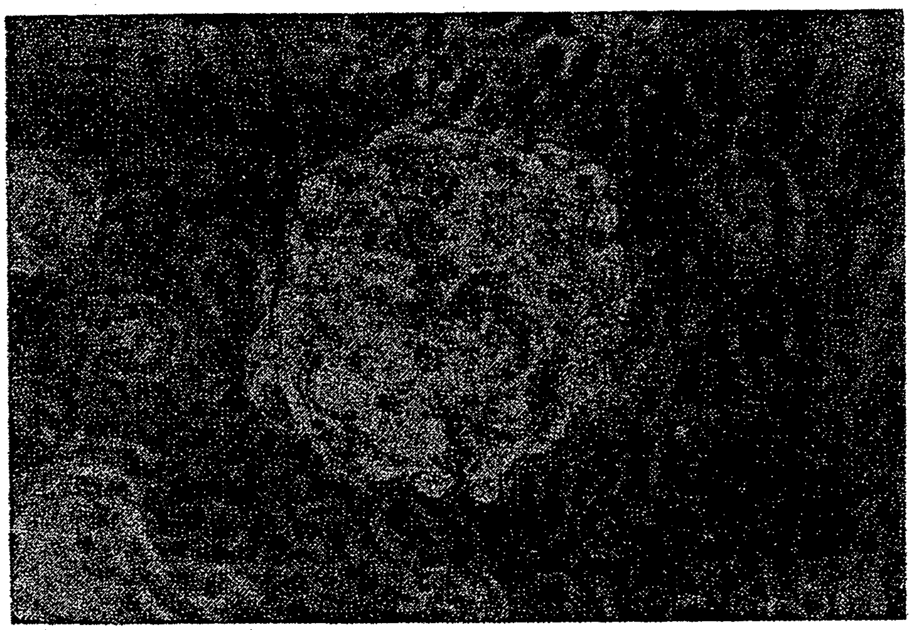


Fig.6.

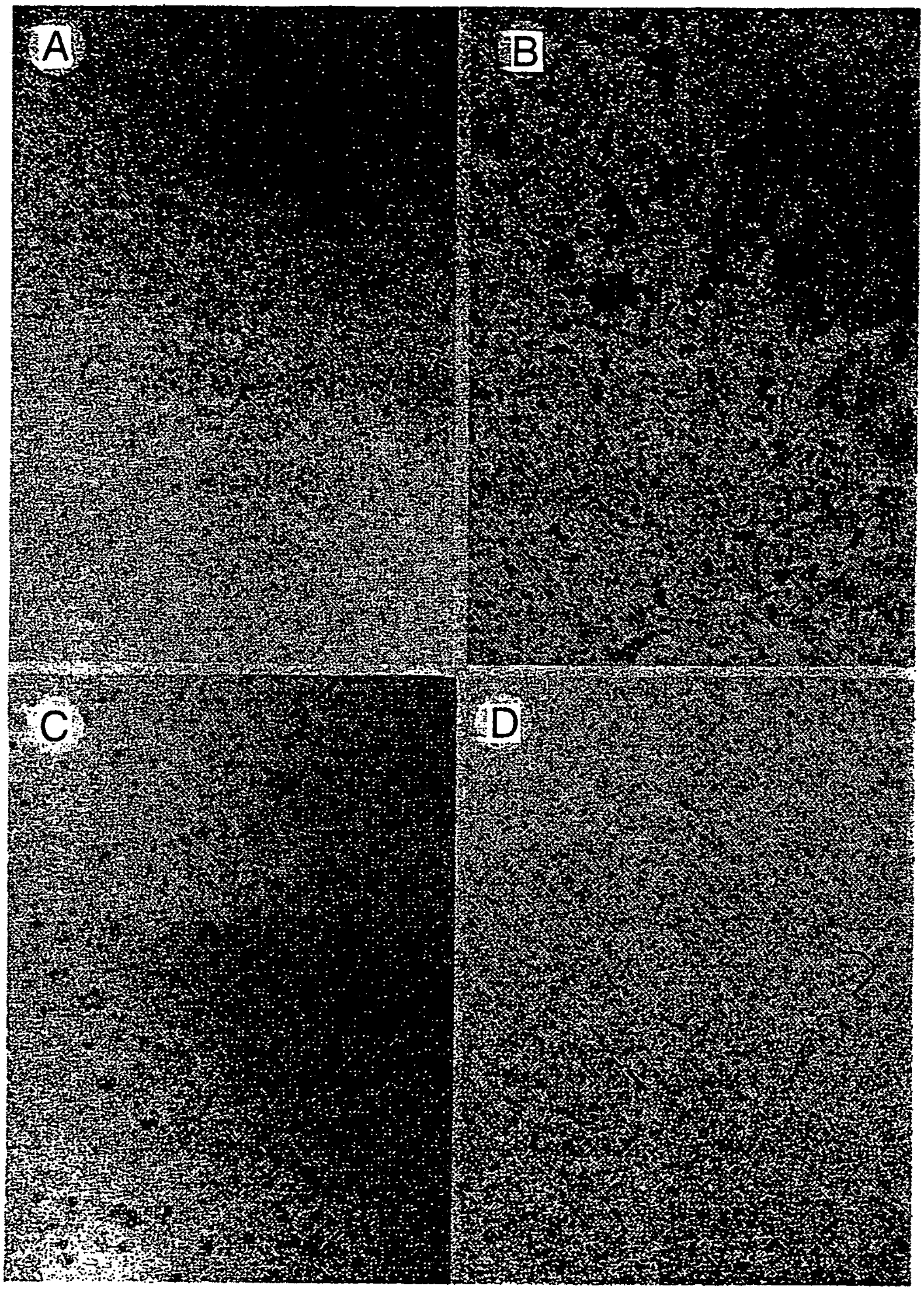
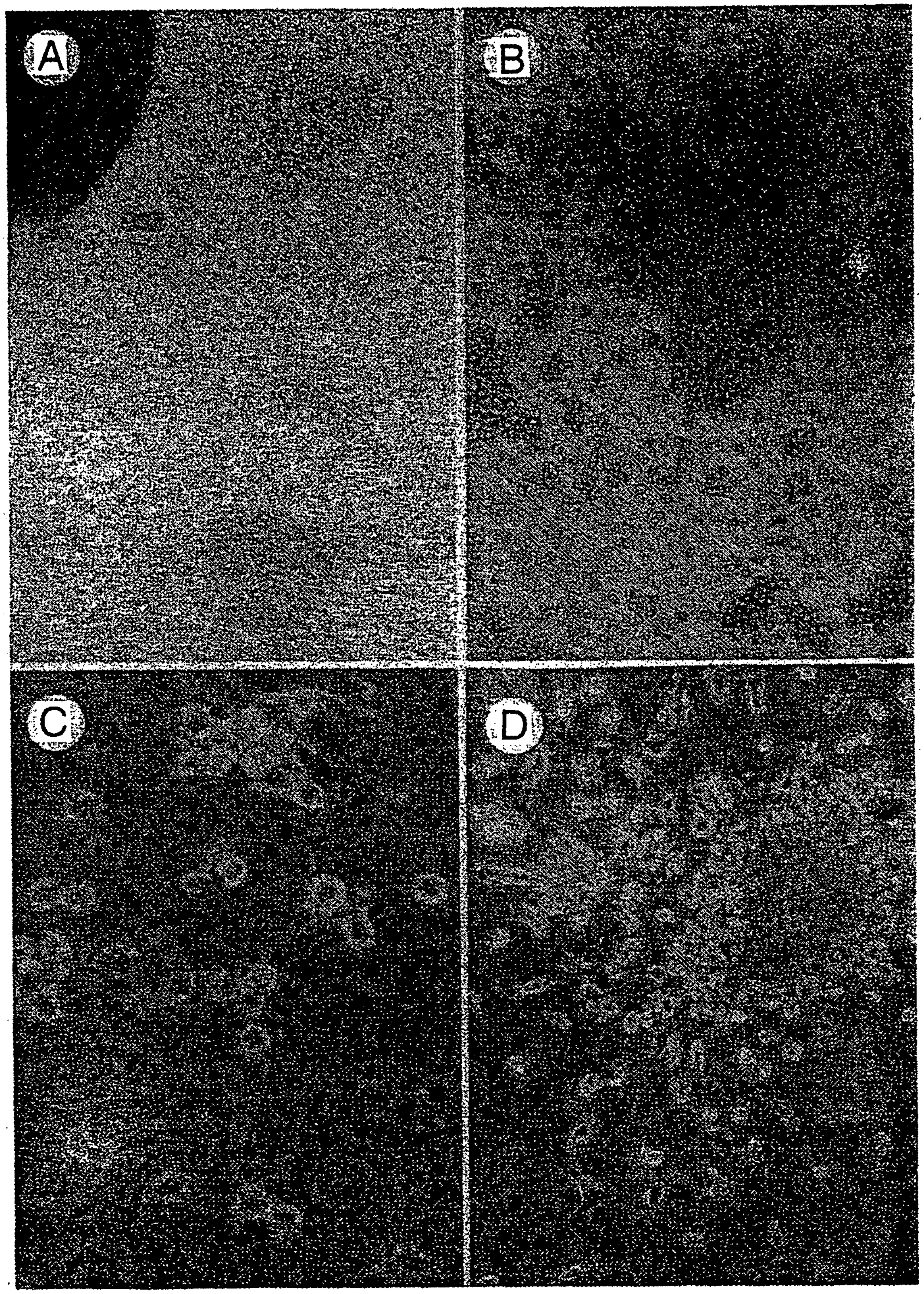


Fig.7.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00624

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.^{5} C12N 5/06, 5/08, C12Q 1/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Electronic Databases as below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC : C12N 5/06, 5/08, C12Q 1/04

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT: WPAT, DIALOG; BIOSIS CHEMICAL ABSTRACTS; CASM.
KEYWORDS : STEM CELLS, EMBRYO, FETAL, GONODAL RIDGE, FEEDER CELLS.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	Y. Matsui et al "Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture", Cell. Vol. 70 (1992) pages 841-847 see abstract.	1-24
Y	S. Saito et al "Bovine embryonic stem cell-like lines cultured over several passages" Roux's Arch Dev. Biol. (1992) Vol. 201 page 134-141 see page 139 column 2 line 11 - page 140 line 39.	1-24

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 1994 (20.01.94)	6 Feb 1995 (06.02.95)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer Ross Osborne Telephone No. (06) 2832404

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	G.B. Anderson "Isolation and use of embryonic stem cells from livestock species". Animal Biotechnology. Vol.3 No 1. (1992) pages 165-175 see whole article.	1-24
Y	WO,A, 90/03432 (ANIMAL BIO-TECHNOLOGY CAMBRIDGE LTD) 5 April 1990 (05.04.90) see page 18 lines 19-23 and claims.	1-24
X	Y. Lallemand et al "An insitu assessment of the routes and extents of the colonisation of the mouse embryo by embryonic stem cells and their descendants" Development Vol. 110 (1990) pages 1241-1248 see page 1246 - page 1247.	22
X	A. Grossler et al "Transgenesis by means of blastocyte derived embryonic stem cell lines" Proc. Natl. Acad. Sci. USA Vol. 83 (1986) page 9065-9069 see whole article.	22
P,Y	WO,A, 94/07997 (US DEPARTMENT OF HEALTH) 14 April 1994 see whole article.	1-24
A	L.E. Urven et al "Differential Gene Expression in Fetal Mouse Germ Cells" Biology of Reproduction Vol. 48 (1993) page 564-574.	1-24
A	B. Wabik-Sliz et al "Culture of mouse germ cells isolated from fetal gonads" Exp. Cell Res. Volume 154 (1984) page 530-536.	1-24

Box I **Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international search report has not established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/AU 94/00624

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9003432	AU	43217/89	BR	8907666	EP	435928
		NZ	230723				
WO	9407997						

END OF ANNEX